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Lethal and sublethal effects of spirotetramat on the mealybug destroyer, *Cryptolaemus montrouzieri*

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ABSTRACT

Spirotetramat is a new systemic insecticide listed in Group 23 of the IRAC mode-of-action classification scheme as an inhibitor of lipid biosynthesis. Previous to its use in IPM programs, it is essential to determine its side effects on selected natural enemies. Herein, lethal and sublethal side effects of spirotetramat on adults and larvae of *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) were evaluated under laboratory conditions by topical application and by ingestion [individuals fed on the citrus mealybug *Planococcus citri* Risso (Hemiptera: Pseudococcidae) previously treated]. Chlorpyriphos and pyriproxyfen, two insecticides commonly used in Spanish citrus, were used as negative controls. Spirotetramat was classified as harmless (OILB: 1) when directly applied on larvae and adults of *C. montrouzieri*, since it did not affect survival, longevity, fecundity, fertility and offspring survival. Contrarily, chlorpyriphos resulted moderately toxic for adults (OILB: 3) due to its effects on fecundity, fertility and offspring survival. Pyriproxyfen resulted harmful (OILB: 4) for larvae due to the acute effect on pupal mortality. When larvae and adults of *C. montrouzieri* were fed with treated prey, spirotetramat was also classified as harmless (OILB: 1). Adults of *C. montrouzieri* fed on pyriproxyfen-treated prey increased their fecundity but eggs hatching was nil. Moreover, the larvae fed on pyriproxyfen-treated prey did not reach the adult stage (OILB: 4). Our results are indicative that spirotetramat may be compatible with augmentative releases of *C. montrouzieri*.

Key words: IPM, side effects, chlorpyriphos, pyriproxyfen, *Planococcus citri*

INTRODUCTION

Coccinellids (Coleoptera: Coccinellidae) are one of the most important groups of predators in biological control programs (Hagen et al. 1999). In Spanish citrus, as elsewhere, coccinellids are a central piece of integrated pest management (IPM) strategies (Jacas and Urbaneja 2010). There are several important citrus pests consumed by naturally occurring coccinellids (Table 1). Among them, the mealybug destroyer *Cryptolaemus montrouzieri* Mulsant is periodically introduced in augmentative releases against the citrus mealybug *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae) (Martínez-Ferrer 2003; Jacas and Urbaneja 2010). *Cryptolaemus montrouzieri* was primarily introduced in a classical biological control program but as it failed to satisfactorily establish in Spain, its current use involves its release from the end of April until July, when *P. citri* gravid females are present, at a dose of three to ten adults per tree repeated every 14-21 days (Jacas et al. 2006; Jacas and Urbaneja 2010). This successful strategy is also used in other Mediterranean countries (Katsoyannos 1997), as well as in areas with a similar climate, such as Australia and California (UC 1991; Smith et al. 1997).

One of the most popular tactics used for the conservation of natural enemies in the Spanish citrus industry has been the use of pesticides with a reduced impact on beneficial arthropods by exploiting either their intrinsic or their ecological selectivity (Croft, 1990). For this reason, new insecticides to be registered in citrus should have a reduced impact on relevant natural enemies. Because *C. montrouzieri* is an important predator in citrus as well as in vineyards and ornamental plants in greenhouses (Daane et al. 2007; Mutsu et al. 2008), the study of the side-effects of new pesticides on this species is necessary. This could be the case of spirotetramat, a new systemic and persistent foliar insecticide. It is a tetramic acid derivative with a novel mode of action that interferes with lipid biosynthesis listed in Group 23 of the IRAC (Insecticide Resistance Action Committee), as spirodiclofen and spiromesifen. It causes the death of immature stages of the target insect from two to ten days after application (Nauen et al. 2006). Spirotetramat is used against *A. aurantii* and other sucking pests, such as aphids, scales (soft and armored), mealybugs, whiteflies, psyllids and selected thrips species in Californian citrus (Grafton-Cardwell et al. 2007; Grafton-Cardwell and Scott 2008). In 2011, spirotetramat was registered in several European countries in different crops. Despite its use in California and its possible use in other citrus areas, the side effects of spirotetramat on coccinellids are still poorly known (Brück et al. 2009).

Therefore, the objective of this study is to assess the lethal and sublethal side-effects of spirotetramat on the immature stages and adults of *C. montrouzieri* resulting from direct contact as well as from ingestion of treated prey (*P. citri*).

MATERIALS AND METHODS

Insecticides.

The concentration of spirotetramat (Movento 150 OD) used in our assays was the maximum proposed by Bayer Crop Science (Valencia, Spain) for the Spanish register, 50 mL/hL. Purified water was used as a positive control in all treatments. The juvenile hormone analogue pyriproxyfen (Juvinal) at 75 mL/hL, and the organophosphate chlorpyrifos (Dursban) at 200 mL/hL, two products commonly used against *P. citri* in Spain (MARM, 2011), were used as negative controls (Table 2). A Potter Spray Tower (Burkard Scientific Ltd©, Uxbridge, UK) was used to apply the insecticides. In all the experiments (direct spray and ingested prey), 2 mL of the corresponding product dilution were sprayed at 150 kPa resulting in a deposit of 1.5 mg/cm^2.

Insects and experimental conditions. Adults and larvae of *C. montrouzieri* used in this assay were obtained from the State Insectary of Valencia (Spain) where they were reared on the citrus mealybug *P. citri* on potato sprouts. Adults were supplied in plastic vials where food, a mixture of honey and agar (50% w/v), was smeared on the inner side of the hermetic lid. First instar larvae were collected from potato sprouts infested by *P. citri*. Prey was obtained from potato sprouts as well.

Environmental conditions in experiments were 25 ± 2ºC, 60 ± 10% RH and a photoperiod of 16:8 h (L:D).

Direct spray of adults. Ten-day-old *C. montrouzieri* adults were used. Six couples per replicate were treated under the Potter tower on a polyethylene mesh (220 × 331 µm mesh) to permit excess liquid evacuation. Ten replicates were conducted for each treatment. Immediately after spraying, one couple per replicate was randomly selected to study their reproductive parameters (fecundity, egg hatching and immature survival of the progeny) and the remaining five couples were used to study adult mortality and longevity following the methods developed by Urbaneja et al. (2008).

To study the reproductive parameters, couples were individually transferred with a fine brush to a Petri dish 90 mm in diameter. Each Petri dish had 4 holes of 7 mm in diameter which were covered by gauze. Couples were kept inside the cage for 12 days and fed with untreated prey. Prey (a mixture of all instars) was offered on a piece of paper (2 × 3 cm), which was used to collect mealybugs from infested sprouts. These pieces of paper were also used by *C. montrouzieri* females as oviposition substrate and were replaced daily and checked under a binocular microscope to ascertain *C. montrouzieri* fecundity and egg hatching. The pieces of paper were subsequently transferred to another Petri dish 60 mm in diameter where egg hatching was measured. Upon hatching, five randomly selected larvae less than 24-hour-old were transferred to a new Petri dish measuring 60 mm in diameter. Larvae were fed *ad libitum* with untreated *P. citri* until reaching the adult stage. Survival was measured.

To study adult mortality and longevity, couples were introduced in a Petri dish 150 mm in diameter. Mortality was recorded daily until day 40 after treatment. Adults were fed a mixture of honey in agar (50% w/v) smeared in a piece of plastic (2 x 3 cm) introduced into the Petri dish (Castañer 1992; Urbaneja et al. 2008).

Direct spray on larvae. Twenty larvae of *C. montrouzieri* less than 24-hour-old (first instar) were treated per replicate under the Potter tower following the method described above. Five replicates were conducted per treatment. Larvae were fed with untreated prey. Immature mortality was evaluated daily until reaching the adult stage. At that moment, ten couples per treatment were further monitored following the previously described protocol to ascertain their reproductive parameters.

Ingestion of treated prey by adults and larvae. *Planococcus citri*-infested potato sprouts were directly sprayed under a Potter Spray Tower using the same conditions as previously described. The sample was also treated on a polyethylene mesh (220 × 331 µm mesh) to permit excess liquid evacuation. The same protocols as above were applied to ascertain the side-effects on untreated adult and larvae when they were fed with treated prey. Only the F1 was fed with treated prey. Descendants were fed with untreated *P. citri*.

Data analysis. Adult survival until day 40 after treatment for both, direct spray and ingested treated-prey experiments, were analyzed by Kaplan-Meier survival analysis. Within the Kaplan-Meier procedure cumulative survival functions (combination of survival time and survival probability) were compared among treatments by Breslow test.

The remaining biological parameters analyzed (fecundity, egg hatching and immature survival) were compared by means of ANOVA. The LSD test was used for mean separation at *P* < 0.05. When significant differences were found (*P*<0.05), the corresponding reduction value (RV) was calculated using the Abbott formula (Abbott 1925). When no significant differences were found the reduction value was assigned as 1. Subsequently, the different reduction values obtained for the same product in an experiment were combined as follows:

$E = RV_{\text{Adult survival}} \times RV_{\text{Fecundity}} \times RV_{\text{Egg hatching}} \times RV_{\text{Immature survival}}$

Where each term represents the percentage reduction in relation to the control for the different parameters considered. The resulting value (E) was interpreted according to IOBC standards (Sterk et al. 1999), which include four categories (1) harmless; (2) slightly harmful, (3) moderately harmful and (4) harmful, which correspond to reductions below 30%, between 31 and 79 %, between 80 % and 99% and higher than 99%, respectively.

RESULTS

Direct spray of adults.

Breslow tests did not reveal significant differences among treatments for the survival of *C. montrouzieri* adults (U = 0.11; P = 0.74) (Table 3 and Figure 1). Fecundity, egg hatching and immature survival were not affected by spirotetramat; however, these three parameters were reduced in the case of chlorpyrifos (Table 3).

Direct spray of larvae.

Spirotetramat did not affect larval and pupal mortality when first instar larvae were directly sprayed (Table 4). Contrarily, pyriproxyfen significantly increased pupal mortality. Similarly, spirotetramat did not affect fecundity during the first ten days of oviposition; however, the fecundity of those specimens treated with pyriproxyfen was nil. Furthermore, egg hatching and immature survival were not affected by spirotetramat.

Effects of treated prey ingestion on adults.

The longevity of *C. montrouzieri* adults was similar irrespective of the type of prey they were fed (U = 1.66; P = 0.20) (Table 5 and Figure 1). Spirotetramat did not affect the fecundity of *C. montrouzieri* females during the first ten days of oviposition, egg-hatching, and survival of the emerged immature stages. On the other hand, pyriproxyfen increased the fecundity of *C. montrouzieri*; however, these eggs did not hatch.

Effects of treated prey ingestion on larvae.

Spirotetramat did not affect larval and pupal mortality of *C. montrouzieri* when larvae fed on treated preys (Table 6). Moreover, adult females obtained from these larvae had a similar fecundity, egg hatching and offspring survival compared with those fed on untreated preys. On the other hand, all immature stages fed on pyriproxyfen-treated prey died.

IOBC Categories.

According to the E-values (%) obtained and following the Standard IOBC categories:

i) The direct spray of adults shows that spirotetramat is harmless (cat. 1), whereas chlorpyrifos, which was used as a negative control, is moderately harmful (cat. 3).

ii) The direct spray of larvae shows that spirotetramat is harmless (cat. 1), whereas pyriproxyfen, the negative control, is harmful (cat. 4).

iii) The treated prey tests on adults and larvae show that spirotetramat is harmless (cat. 1) to both stages, whereas pyriproxyfen is harmful (cat. 4).

DISCUSSION

Our results show that spirotetramat could be compatible with augmentative releases of the coccinellid *C. montrouzieri*. Following the recommendations of the International Organization of Biological Control (IOBC) (Sterk et al. 1999), spirotetramat can be categorized as harmless (cat. 1) to *C. montrouzieri* because laboratory trials showed no harm against this species. These results are based on both lethal (survival) and sublethal (fecundity, egg hatching and offspring survival) effects of on adults and immature stages of *C. montrouzieri* when the insecticide was directly applied as well as when it was ingested through treated prey.

According to our data, spirotetramat is much less toxic than the two conventional insecticides used as control in this study. Consequently, its use could be considered in IPM programs where coccinellids are key natural enemies as it is the case of Spanish citrus, where pyriproxyfen and chlorpyrifos are widely used against *A. aurantii* (Tena et al. 2011). However, as shown herein and in previous studies these insecticides are toxic for *C. montrouzieri*, as well as for other natural enemies (Jacas and Urbaneja 2010).

In our study, we used pyriproxyfen and chlorpyrifos as negative controls because of its known toxicity against different stages of *C. montrouzieri* (Pascual and Urbaneja 2006).

Our results allow us to categorize the growth regulator insecticide, pyriproxyfen, as harmful (cat.4) for larvae and adults of *C. montrouzieri*. Larvae either treated with this insecticide or fed with treated prey reached the pupal stage but they died during this stage and those that survived were sterile. Contrarily, adults fed on treated prey did not die and indeed increased their fecundity. Nevertheless, their eggs did not hatch. Thus, pyriproxyfen affected all developmental stages of *C. montrouzieri* but its effect was different in immature stages and adults. The effect of pyriproxyfen on the fecundity and egg hatching has been previously described for *C. montrouzieri* (Hatting and Tate 1995; Franco et al. 2004), as well as for other coccinellids (Mendel et al. 1994; Grafton-

Cardwell and Gu 2003). Only Cloyd and Dickinson (2006) observed that pyriproxyfen was harmless to the adult stage of *C. montrouzieri*. Nonetheless, their study was solely based on the mortality of *C. montrouzieri* adults 48h after the spray. Therefore, our results confirm the importance of estimating sublethal effects in this kind of studies (Desneux et al 2007). Galvan et al. (2005) also observed that spinosad did not affect the survival of *Harmonia axyridis* (Pallas) adults, but reduced female fertility.

In a previous study, Boyero et al. (2005) demonstrated that chlorpyrifos, contrary to pyriproxyfen, affected the survival of *C. montrouzieri* adults. For this reason, we decided to include chlorpyrifos as a negative control in the adult direct spray test. In general, our study shows that chlorpyrifos is moderately toxic (cat. 3) for adults of *C. montrouzieri*. Although our results showed that chlorpyrifos did not affect adult survival, it reduced fecundity and fertility more severely than pyriproxyfen. Bellows and Morse (1988) also studied the side effects of chlorpyrifos on *C. montrouzieri.* They found that the effect of the residues of chlorpyrifos on the survival of *C. montrouzieri* adults was lower than that of other insecticides (carbaryl, ethidathion, and parathion) but they did not study the sublethal effects of these biocides. For future studies on the side effects of pesticides on coccinellid adults by direct application, we would recommend the use of chlorpyrifos instead of pyriproxyfen as a negative control. Moreover, if the purpose is to determine the side effects of chlorpyrifos we entirely encourage studying its sublethal effects.

Based on the side effects observed in our study, spirotetramat can be included in IPM programs where *C. montrouzieri* is a key natural enemy, as citrus, vineyards, and ornamental plants. In citrus, where *A. aurantii* is a key pest, spirotetramat is an alternative insecticide to chlorpyrifos and pyriproxyfen which are widely used. The use of spirotetramat against the first generation of *A. aurantii* at the end of spring is compatible with augmentative releases of *C. montrouzieri* adults in the field. However, it would be desirable to extend the information of its side effects to other key beneficial insects in citrus, particularly on a relevant parasitoid such as *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) and a relevant predatory mite such as *Euseius stipulatus* (Athias-Henriot) (Acari: Phytoseiidae).

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Table 1. Important pests and their coccinellid predators (Coleoptera: Coccinellidae) occurring in citrus in Spain.

Target pest	Coccinellid predator	Reference
Aphis spiraecola Patch and *A. gossypii* Glover (Hemiptera: Aphididae)	*Scymnus* sp. *Propylea quatuordecimpunctata*, L.,	1, 2
Aonidiella aurantii (Maskell) (Hemiptera: Diaspididae)	*Rhyzobious lophantae* (Blaisdell)	2, 3
Tetranychus urticae Koch (Acari: Tetranychidae)	*Stethorus punctillum* Wiese	2, 4
Icerya purchasi Maskell (Hemiptera: Margarodidae)	*Rodolia cardinalis* Mulsant	2, 5
Planococcus citri (Risso) (Hemiptera: Pseudococcidae)	*Cryptolaemus montrouzieri* Mulsant	2, 5

References: 1) Michelena and Sanchis 1997, 2) Urbaneja et al. 2011, 3) Alvis-Dávila et al. 2002, 4) Abad-Moyano et al. 2009, 5) Jacas and Urbaneja 2010

Table 2. Products and doses used as negative controls for each case.

Assay	Negative control	Dose (mL/ hL)
Direct spray on adults	chlorpyrifos 48%	200
Direct spray on larvae	pyriproxyfen 10%	75
Ingestion of treated prey	pyriproxyfen 10%	75

Table 3. Cumulate survival (%), fecundity (eggs/female during 10 days), egg hatching (%), and F_1 survival (%) of *Cryptolaemus montrouzieri* adults exposed to different products by direct spray and water-treated control. Reduction factors for each parameter appear in brackets and the IOBC category (IOBC cat.) corresponding to the total reduction (*E*) are shown in the last row.

	Chlorpyrifos	Spirotetramat	Control	Statistics
Cumulative survival[a]	28.4 ± 0.1a [1.00]	28.6 ± 1.0a [1.00]	30.0 ± 0.1a	
Fecundity	64.4 ± 14.2b [0.60]	82.6 ± 10.6ab [1.00]	107.0 ± 6.1a	$F = 3.8$; df = 2, 29; $P = 0.0332$
Egg hatching	37.2 ± 6.5b [0.53]	62.3 ± 5.5a [1.00]	70.4 ± 4.9a	$F = 9.1$; df = 2, 89; $P = 0.0003$
Immature survivals	50.0 ± 7.6b [0.57]	86.6 ± 5.5a [1.00]	88.0 ± 4.1a	$F = 13.1$; df = 2, 89; $P < 0.0001$
E (%) (IOBC cat.[b])	81.8 **(3)**	0.0 **(1)**		

[a] Within a row, data followed by a different letter are significantly different ($P < 0.05$, LSD test)

[b] Categories OILB: 1: harmless; 2: slightly harmful; 3: moderately harmful; 4: harmful.

Table 4. Larval mortality (%), pupal mortality (%), fecundity (eggs/female during 10 days), egg hatching (%), and F_1 survival (%) of *Cryptolaemus montrouzieri* larvae exposed to different products by direct spray and water-treated control. Reduction factors for each parameter appear in brackets, and the IOBC category corresponding to the total reduction (*E*) are shown in the last row.

	Pyriproxyfen	Spirotetramat	Control	Statistics
Larval mortality[a]	6.0 ± 1.8a [1.00]	2.0 ± 1.2a [1.00]	4.8 ± 1.5a	$F = 1.7$; df = 2, 14; P=0.23
Pupal mortality	89.9 ± 2.3a [0.11]	2.0 ± 1.2b [1.00]	4.8 ± 1.5b	$F = 779,8$; df = 2, 14; $P < 0.0001$
Fecundity	0.0 ± 0.0b [0.00]	149.2 ± 8.2a [1.00]	170.9 ± 10.92a	$F = 139.2$; df = 2, 29; $P < 0.0001$
Egg hatching	–	86.6 ± 2.8a [1.00]	93.0 ± 1.8a	$F = 3.5$; df = 1, 59; $P = 0.07$
Immature survivals	–	60.0 ± 5.8a [1.00]	63.3 ± 6.3a	$F = 0.15$; df = 1, 59; $P = 0.7$
E (%) (IOBC cat)[b]	100.0 **(4)**	0.0 **(1)**		

[a] Within a row, data followed by a different letter are significantly different ($P< 0.05$, LSD test)

[b] Categories OILB: 1: harmless; 2: slightly harmful; 3: moderately harmful; 4: harmful.

Table 5. Cumulate survival (%), fecundity (eggs/female during 10 days), egg hatching (%), and F_1 survival (%) of *Cryptolaemus montrouzieri* adults exposed to different products by ingestion of treated prey (*Planococcus citri*) and water-treated control. Reduction factors for each parameter appear in brackets, and the IOBC category corresponding to the total reduction (E) are shown in the last row.

	Pyriproxyfen	Spirotetramat	Control	Statistics
Cumulative survival[a]	33.1 ± 0.1a [1.00]	34.1 ± 0.1a [1.00]	32.1 ± 1.7a	
Fecundity	183.5 ± 18.3a [1.00]	95.5 ± 18.1b [1.00]	102.1 ± 21.3b	$F = 6.42$; df = 2, 29; $P = 0.0052$
Egg hatching	0.0 ± 0.0b [0.00]	79.6 ± 4.3a [1.00]	76.0 ± 2.6a	$F = 233.4$; df = 2, 89; $P < 0.0001$
Immature survivals	-	88.6 ± 3.1a [1.00]	88.6 ± 2.6a	$F = 0.0$; df=1, 59; $P = 1.0$
E (%) (IOBC cat)[b]	100.0 **(4)**	0.0 **(1)**		

[a] Within a row, data followed by a different letter are significantly different ($P < 0.05$, LSD test)

[b] Categories OILB: 1: harmless; 2: slightly harmful; 3: moderately harmful; 4: harmful.

Table 6. Larval mortality, (%), pupal mortality (%), fecundity (eggs/female during 10 days), egg hatching (%), and F_1 survival (%) of *Cryptolaemus montrouzieri* larvae exposed to different products by ingestion of treated prey (*Planococcus citri*) and water-treated control. Reduction factors for each parameter appear in brackets, and the IOBC category corresponding to the total reduction (*E*) are shown in the last row.

	Pyriproxyfen	Spirotetramat	Control	Statistics
Larval mortality[a]	78.0 ± 0.4a [0.23]	2.0 ± 1.2b [1.00]	3.0 ± 0.3b	F = 177.5; df = 2, 14; P < 0.0001
Pupae mortality	100.0 ± 0.0a [0.00]	17.0 ± 4.8b [1.00]	5.0 ± 3.1b	F = 466.6; df = 2, 14; P < 0.0001
Fecundity	–	134.7± 10.6a [1.00]	149.4 ± 13.7a	F = 0.72; df = 1, 59; P = 0.41
Egg hatching	–	88.3 ± 2.62a [1.00]	92.0 ± 3.4a	F = 0.71; df = 1, 59; P = 0.4
Immature survivals	–	57.3 ± 6.2b [0.80]	72.0 ± 3.6a	F = 4.1; df = 1, 59; P = 0.048
E (%) (IOBC cat)[b]	100.0 **(4)**	0.0 **(1)**		

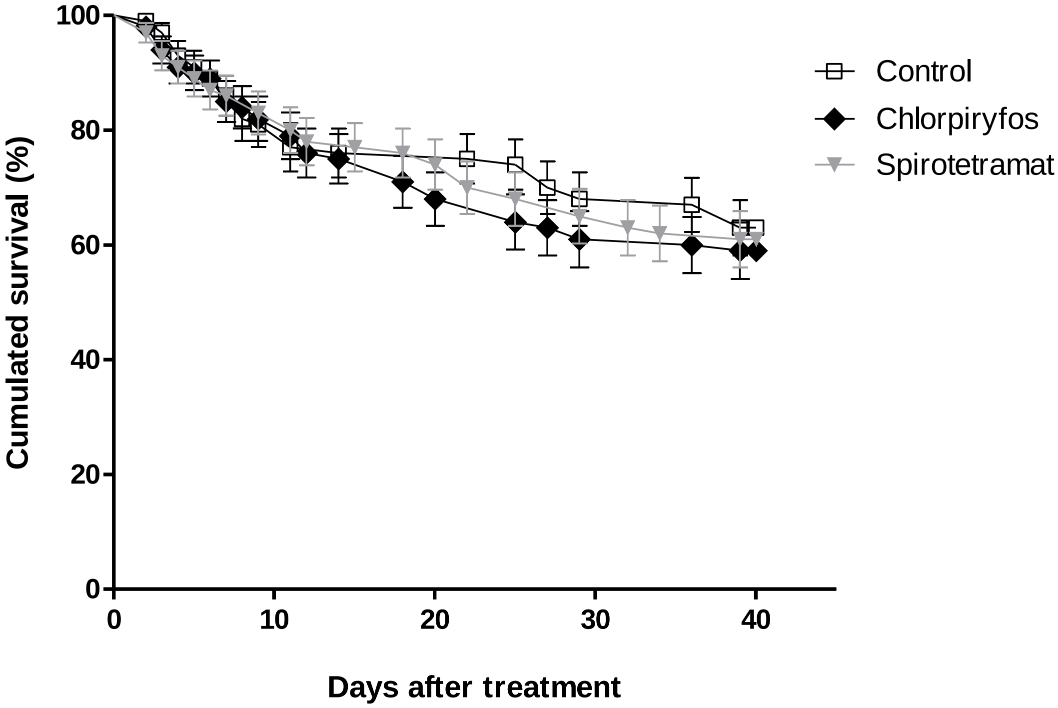
[a] Within a row, data followed by a different letter are significantly different (*P*< 0.05, LSD test)

[b] Categories OILB: 1: harmless; 2: slightly harmful; 3: moderately harmful; 4: harmful.

Figure legends

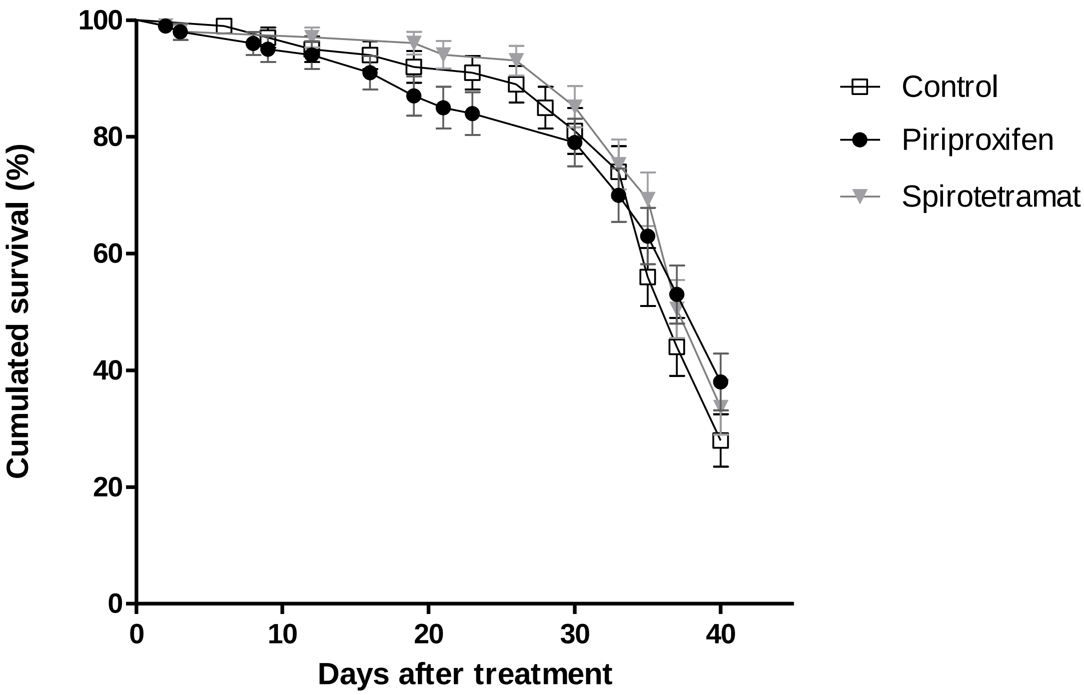
Figure 1. Adult survival of *Cryptolaemus montrouzieri* when they were a) direct sprayed and b) fed on treated prey.

a)



Cumulated survival (%)
100
80
60
40
20
0
0
10
20
30
40
Days after treatment
Control
Chlorpiryfos
Spirotetramat

a)



Cumulated survival (%)
100
80
60
40
20
0
0
10
20
30
40
Days after treatment
Control
Piriproxifen
Spirotetramat

b)

Fig 1.